THE COMPLEX OF ATP-DEPENDENT DEOXYRIBONUCLEASE FROM *BACILLUS SUBTILIS* WITH DNA AND ITS THERMOSTABILITY

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1. Introduction

We have earlier reported the existance of an ATP-dependent DNAase from *B. subtilis*, its partial purification and its properties [1, 2]. Among a few *rec*-mutants of *B. subtilis* one (342) was found with 5–10 times lower activity of the DNAase in the extracts. A number of reverse mutations were obtained from the 342 strain selected for their stability to the mitomycin C [3]. All revertants except one had normal ATP-dependent DNAase activities. Together with other known data [4–7] the above mentioned results suggest an important role of the DNAase in DNA recombination. Therefore, it seemed interesting to find out if the enzyme could form a complex with the DNA and if it did to study the influence of the DNA and ATP on the enzyme stability.

2. Methods

All experiments were carried out with the ATP-dependent DNAase from the *B. subtilis* SB 25 *his,ind* purified to the stage IV (gel filtration through Sephadex G-200 column; see [1]). Conditions of bacterial growth, purification and testing of the enzyme and isolation of labelled and unlabelled DNA from *B. subtilis* were as described previously [1, 2]. The procedure of the bacteriophage T2 [^{14}C]DNA isolation was as in [8]. The specific radioactivity of the [^{14}C]DNA's was about 10,000 cpm/μg.

3. Results and discussion

3.1. *Formation of the enzyme–DNA complex*

Our first aim was to find out whether the ATP-dependent DNAase is able to form a stable complex with DNA substrate. The enzyme, [^{14}C]DNA, and their mixture after 5 min preincubation were centrifuged separately through glycerol gradients (10–35%) (fig. 1). It was observed that the enzyme with the DNA (curves 3, 4) moved faster than the free [^{14}C]DNA (curve 1) or the free enzyme (curve 2). A difference was noticed in the shape of curve 3 (DNA–enzyme complex tested by DNA radioactivity) and curve 4 (DNA–enzyme complex tested by enzyme activity without additional [^{14}C]DNA). This difference may be due to heterogeneity of the [^{14}C]DNA as a result of which the shorter DNA molecules bind relatively greater amount (by weight) of the enzyme. This explanation may be correct only if each DNA molecule binds only one (or two) molecule of the enzyme. Some of the ATP-dependent DNAase did not complex with DNA as some enzymatic activity (curve 5, tested with additional [^{14}C]DNA) could be seen in the fraction of the glycerol gradient coinciding with the position of the free enzyme. At higher enzyme/DNA ratios the amount of the unbound enzyme fraction increased (results of this experiment are not shown here).

Thus, the *B. subtilis* ATP-dependent DNAase forms a complex with DNA having a higher sedimentation rate than free DNA or enzyme. However, it was not

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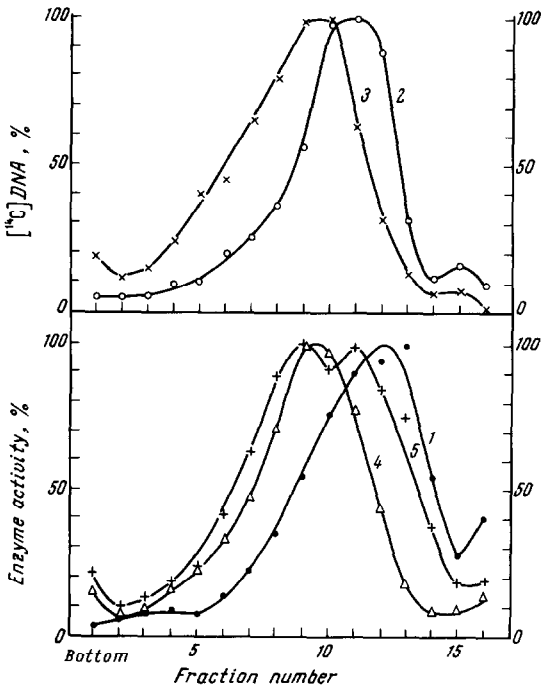

Fig. 1. Distribution of ATP-dependent DNAase (curve 1), [^{14}C]DNA (curve 2), and their mixture (curves 3–5) in glycerol gradients. 80 μg of the [^{14}C]DNA, 2 mg of the enzyme preparation, and their mixture in 0.2 ml of 0.02 M Tris-HCl, pH 7.5, 1.0 mM EDTA, 5.0 mM 2-mercaptoethanol after 5 min preincubation at 37° were layered on a 10–35% linear glycerol gradient (4.8 ml) in the same buffer and spun for 4 hr at 40,000 rpm in an RKS-50T rotor (ultracentrifuge UCP-60, USSR) at 4°. Aliquots of the enzyme–[^{14}C]DNA gradient fractions were tested for DNA radioactivity (curve 3), the enzymatic activity with endogenous [^{14}C]DNA (curve 4) or for enzymatic activity with an additional 5 μg [^{14}C]DNA in each test tube (curve 5). Enzymatic activity (see [1]) or DNA radioactivity of the most active fractions were taken for 100%.

clear whether the enzyme–DNA complex dissociated or not after each cleavage of a phosphodiester bond. To clarify this point we checked whether the complexed ATP-dependent DNAase is able to go over to another DNA molecule in the presence or absence of ATP (table 1).

To form the complex the enzyme was preincubated for 3 min with the [^{14}C]DNA and ATP. After cooling, a 5-fold excess of unlabelled DNA was added, and incubation proceeded for another 17 min. The amount of [^{14}C]DNA digested (no. 3) was not much lower than when no unlabelled DNA was added (no. 1). At the same time addition of the unlabelled DNA together with [^{14}C]DNA before preincubation gave rise to considerable decrease of digested [^{14}C]DNA which was roughly proportional to dilution of the [^{14}C]DNA solution with unlabelled DNA (no. 2). When the unlabelled DNA was added before the preincubation with the enzyme and ATP and [^{14}C]DNA before the incubation, a great decrease of solid soluble radioactivity was observed (no. 4). Thus it is clear that the enzyme–DNA complex did not dissociate after each phosphodiester bond cleavage.

It is interesting to note that the enzyme–DNA complex formed in the absence of ATP was less stable than the one formed in the presence of ATP. Although this complex was not dissociated during glycerol gradient centrifugation and therefore could be distinguished from the free DNA or free enzyme by an increased sedimentation rate, the enzyme was still able to go over from one DNA molecule to another (table 1, nos. 5–7).

3.2. *Influence of DNA and ATP on enzyme stability*

Many enzymes are known to be stabilized after binding with substrates or after initiation of the enzymatic reaction. Generally such stabilization is accounted for by a structural alteration of the enzyme. Therefore, it was interesting to see if complex formation with DNA or addition of ATP could cause stabilization of the ATP-dependent DNAase.

We chose incubation at elevated temperatures as an inactivating agent. To determine the temperature range of enzyme inactivation reaction tubes with the enzyme were preincubated for 5 min at different temperatures, cooled, [^{14}C]DNA and ATP were added, and incubated 30 min at 37° to determine the remaining enzymatic activity. Up to 40° no activity changes were observed and then it gradually decreased to zero at 55° (fig. 2, curve 2). The preincubation at all temperatures tested did not affect the background activity of the enzyme determination without ATP (curve 3). In other tubes the enzyme was preincubated with [^{14}C]DNA, and before incubation ATP was added (curve 1). The protective effect of DNA was observed. In all experiments preincubation with DNA at 40–50° caused some stimulation of the enzyme activity. The stabilization of the enzyme took place after preincubation not only with *B. subtilis* DNA, but also with phage T2 DNA

Table 1
Absence of an equilibrium between complexed and free DNA.

Expt. no.	Preincubation			Incubation			Acid-soluble [^{14}C]DNA (%)
	DNA (μg)		ATP (μM)	DNA (μg)		ATP (μM)	
	Labelled	Unlabelled		Labelled	Unlabelled		
1	5	–	10	–	–	–	100
2	5	25	10	–	–	–	18
3	5	–	10	–	25	–	67
4	–	25	10	5	–	–	6
5	5	25	–	–	–	10	17
6	5	–	–	–	25	10	19
7	–	25	–	5	–	10	16

Test tubes contained standard reaction mixture (0.5 ml); 0.05 M Tris-HCl, pH 9.0, 0.01 M $MgCl_2$, 1.0 mM EDTA, 5 mM 2-mercaptoethanol and 50 μg of the enzyme and ATP and DNA as indicated. Preincubation time was 3 min and incubation time was 17 min (nos. 1–3) or 20 min (nos. 4–7).

(fig. 3). Thus, preincubation of the ATP-dependent DNAase with DNA accompanied by complex formation stabilized the enzyme against thermoinactivation.

In the following experiments the effect of enzyme preincubation with ATP was studied. It was rather surprising to find that 5 min preincubation in the presence of ATP caused destabilization of the enzyme (fig. 4). It will be seen that inactivation of the enzyme in the presence of ATP occurs at 5–10° lower temperature than with the enzyme alone. The significance of this result is still obscure.

Thus, it is clear that *B. subtilis* ATP-dependent DNAase forms with DNA a complex the stability of which greatly increases in the presence of ATP, i.e. after incubation of DNA digestion. In the presence of ATP the complex is so stable that the enzyme molecule can hardly go over, if at all, to another DNA molecule. Perhaps after initiation of the enzymatic reaction the enzyme molecule digests one chain of a duplex using the other chain as a template. The results of this work are in agreement with those of Winder and Sastry [9] who showed that the ATP-dependent DNAase from *Mycobacterium smegmatis* formed a complex with DNA, which the authors separated from the free enzyme and DNA by a millipore filtration

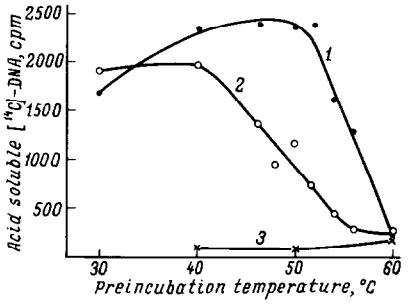

Fig. 2. Effect of the *B. subtilis* DNA on heat inactivation of the ATP-dependent DNAase. 50 μg of enzyme preparation with 2.5 μg of [^{14}C]DNA (curves 1 and 3), or without DNA (curve 2) in standard reaction mixtures (see table 1) were preincubated for 5 min without ATP at different temperatures and cooled. Then ATP was added to 50 μM (curves 1 and 2) or not added (curve 3), and mixtures were incubated at 37° for another 30 min and the remaining enzymatic activity determined.

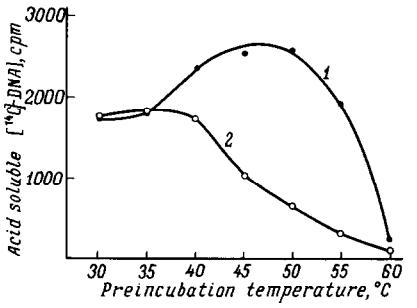

Fig. 3. Effect of the T2 DNA on the heat inactivation of the ATP-dependent DNAase. Conditions of the experiment are the same as in fig. 2. Enzyme was preincubated with (1) or without (2) [^{14}C]DNA.

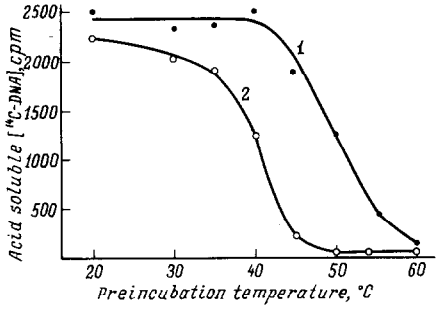

Fig. 4. Effect of ATP on the heat inactivation of the ATP-dependent DNAase. 50 μg of the enzyme preparation with 50 μM ATP (curve 2), or without ATP (curve 1) in a standard reaction mixture (see table 1) were preincubated for 5 min at various temperatures and cooled, 2.5 μg of [^{14}C]DNA was added and test tubes were incubated at 37° for another 30 min and the remaining enzyme activity determined.

technique. However, they were unable to find this complex in the absence of ATP. This may be due to some differences in the experimental techniques or sources of the enzymes. The fact that *B. subtilis* ATP-dependent DNAase is able to form a complex with DNA in the absence of ATP is shown not only by sedimentation analysis but also by the fact that DNA alone stabilizes the enzyme.

Almost all of these properties of ATP-dependent DNAase resemble the properties of the template enzymes such as RNA or DNA polymerases, and they may be important in the DNA recombination process.

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